APPENDIX A

Human PATHOLOGY

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STATEMENT OF PURPOSE: HUMAN PATHOLOGY is designed to bring to the laboratory and clinical physician, and to the student, authoritative information of clinicopathologic significance to human disease. Its primary goal is the presentation of information drawn from morphologic and clinical laboratory studies having direct relevance to the understanding of diseases of humans. Accordingly, it is intended that the journal embrace articles concerned with morphologic and clinicopathologic observations, reviews of a disease or group of diseases, analyses of problems in pathology, significant collections of case material, and new advances in concepts or techniques of value in the analysis and diagnosis of disease. Theoretical and experimental pathology and molecular biology pertinent of the diseases of humans will be included. The journal articles will be of interest to all physicians engaged in the clinical and laboratory practice of medicine as well as to teachers of pathology and clinical medicine. They will be critical and authoritative. Above all, they will embody a point of view.

HUMAN PATHOLOGY is covered in Index Medicus, Excerpta Medica, Current Contents/Life Sciences, Current Contents/Clinical Medicine, Science Citation Index, BIOSIS, and ISI/BIOMED.

In Situ Hybridization Techniques for the Analysis of Gene Expression: Applications in Tumor Pathology

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In situ hybridization (ISH) has emerged over the past decade as an extraordinarily sensitive technique for the detection of gene expression at the cellular level. Advances in probe preparation and labeling methods have facilitated the transfer of this technology from the research laboratory to the clinical arena. In contrast to immunohistochemistry, which is dependent on the protein content of cells, ISH analyses permit the identification of cells on the basis of their contents of specific messenger RNAs (mRNAs) encoding the products of interest. These methods provide a critical approach for the analysis of heterogeneity in tumors that typically contain cells at different phases of neoplastic progression and at multiple levels of differentiation and functional activity. In situ hybridization methods have been of particular value for studies of mRNAs encoding oncogenes, hormones, secretory proteins, cytokines, and a wide variety of other cellular products. Advances in ISH technology, including polymerase chain reaction (PCR) based methods, offer particular promise for examining genes with low levels of expression at the cellular level. HUM PATHOL 25:580–585. Copyright © 1994 by W.B. Saunders Company

In situ hybridization (ISH) is a histochemical method that employs the techniques and reagents of molecular biology in much the same way that immunohistochemistry uses the methods of immunology (Fig 1).[1] This technology is based on the principle that cell- and tissue-bound RNA and DNA sequences will hybridize with labeled probes of complementary sequence. Sites of hybridization can then be visualized microscopically. Although ISH was developed a quarter of a century ago,[2] this technique did not gain widespread acceptance until relatively recently. Over the last decade, however, there have been numerous refinements of ISH technology and many applications in basic research and in diagnostic pathology laboratories.

As compared with other molecular technologies, ISH provides a high degree of spatial resolution that permits the localization of single-copy genes to individual chromosomes,[3] the identification of translocations and other chromosomal abnormalities,[4,5] and the study of gene expression at the cellular and subcellular levels.[6,7] In addition, these methods have been used extensively for the identification of viruses and other microorganisms.[8-11] This review will focus, however, on the use of these methods for studies of gene expression, particularly in neoplasms.

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A major advantage of ISH for analysis of gene expression is its ability to localize messenger RNAs of interest to specific cells in heterogeneous tissues. As a result, this technology can provide data that expand and refine the results of Northern blot analyses. In addition, ISH enables the investigator to perform multiple analyses on small biopsy samples. As noted by Wilcox, a digest from such a small sample may yield sufficient RNA for one or two Northern blots, whereas a tissue block can yield potentially hundreds of sections that can be used for a variety of hybridizations with different probes.[12] An additional advantage is that intervening sections also may be used for routine morphological study, immunohistochemistry, and quantitative DNA analyses.

METHODOLOGY

The success of ISH depends on numerous factors, including the numbers of copies of DNA and RNA expressed in the cells of interest and the extent to which they are preserved or fixed. In addition, the type of probe, hybridization and stringency conditions, method of probe labeling (radioactive *v* nonradioactive), and the techniques of signal detection are crucial factors.

Many fixatives have been evaluated for ISH analyses.[13] Most studies have showed that messenger RNA (mRNA) is preserved optimally by cross linking aldehyde fixatives. Precipitating fixatives, such as Carnoy's, Bouin's, and Zenker's, generally provide less retention of mRNA than does formaldehyde. Buffered 4% paraformaldehyde has been the fixative of choice in most studies. Tissue should be fixed in 4% paraformaldehyde immediately after removal in order to prevent breakdown of RNA by endogenous ribonucleases. Following fixation the tissues can be transferred to 30% sucrose to remove residual fixative and to diminish freeze artifacts on subsequent quenching and storage of tissues in liquid nitrogen prior to the preparation of frozen sections.[14] Cytocentrifuge preparations and fresh frozen sections also have been used successfully for ISH analyses. In some instances formalin-fixed, paraffin-embedded tissues prepared for routine diagnostic use have given excellent results for the demonstration of some abundantly expressed mRNAs.

Pretreatment of tissue sections with detergents (such as Triton X-100) and proteolytic enzymes (trypsin and proteinase K) just before hybridization facilitates the interactions of the probes with their target nucleic acids.[15] In general, the longer the primary fixation time, the greater is the need for proteolytic digestion.

Many types of probes have been used for ISH analyses, including nick-translated or randomly-primed double-stranded DNA probes, single-stranded complimentary (c) DNA probes prepared in the M13 vector, and single-stranded antisense or complementary RNA (cRNA) probes.[14,15] Over the course of the past several years the choice of probe has shifted from

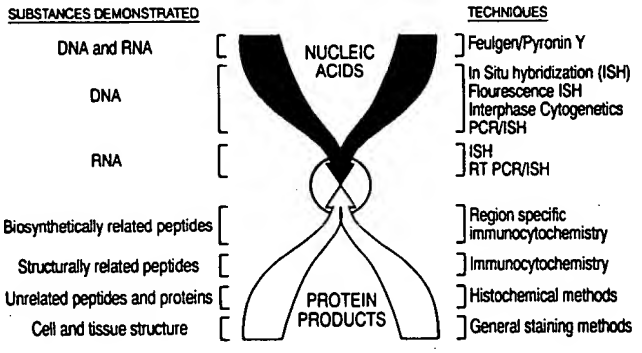

FIGURE 1. Evolution of technology for the evaluation of genes and gene expression at the cellular level. At the nucleic acid level technology has advanced from the use of histochemical stains, such as the Feulgen reaction, for the demonstration of DNA and pyronin Y for RNA to ISH. Defined segments of DNA can be shown in interphase nuclei and in chromosome spreads by fluorescence ISH (FISH) or nonfluorescence ISH methods. Foreign DNA (eg, viruses and other organisms) also can be demonstrated effectively by ISH. Amplification of DNA by the polymerase chain reaction (PCR) can be combined with ISH (PCR-ISH) to provide markedly enhanced sensitivity (in situ PCR). Both native and foreign (eg, viruses) RNAs of interest can be shown by ISH using a variety of different probes and labeling formats. In situ PCR can be extended to studies of RNA after the in situ synthesis of the corresponding cDNA through the use of reverse transcriptase (RT PCR/ISH). At the protein level technology has advanced from the use of empiric staining methods to the use of monoclonal antibodies reactive with biosynthetically related peptides (region specific immunocytochemistry). The combination of ISH methods for nucleic acids and immunocytochemical methods for proteins can now provide maximal information on genes and gene expression at the level of the single cell (center circle). (Adapted and reprinted with permission from DeLellis RA, Wolfe HJ: New techniques in gene product analysis. Arch Pathol Lab Med 111:620-627, Copyright 1987; American Medical Association.[70])

double-stranded DNA probes to cRNA (riboprobes) and synthetic oligonucleotide probes. As compared with nick-translated probes, cRNA probes can be labeled to a higher specific activity and have greater thermal stability; moreover, the lack of back-hybridization is another significant advantage of riboprobes.[12,16] Nonhybridized, single-stranded RNA probes can be removed by treatment with RNAse. Antisense probes are prepared in transcription vectors that contain promotors located upstream from multiple cloning sites. The DNA of interest is inserted into the cloning site and can be transcribed in the presence of labeled nucleotides to provide sense or antisense probes. A sense probe will have the same 5′ to 3′ orientation as the transcribed mRNA. It will not therefore hybridize to the mRNA but will hybridize to one of the DNA strands, provided that the DNA has been denatured or melted before the reaction.

Single-stranded DNA probes produced by amplified primer extension (APEX) labeling with Taq polymerase also have been used in ISH formats with excellent results.[17] In this procedure conventional PCR is used to produce the DNA fragment of interest, which is then used as a template for the probe labeling reaction. This step involves repeated cycles of primer extension by Taq polymerase in the presence of labeled nucleotide precursors with a single antisense primer. As a result, only the antisense strand will be labeled. The studies of Brooks and associates have shown that probes produced by this method possess sufficient sensitivity to detect low abundance neuropeptide heteronuclear RNA by ISH.[17]

Synthetic oligonucleotide probes, generally 20 to 50 mers have several advantages over cloned DNA probes. The use of oligonucleotide probes does not require detailed expertise with basic molecular technologies because the probe is not cloned and labeling can be carried out in a single step by either 3′ or 5′ labeling.[18] Secondly, oligonucleotide sequences can be constructed from published cDNA maps and can be produced relatively inexpensively through commercial sources. A major advantage of these probes is that they can be used to achieve high levels of specificity under conditions of high stringency. Moreover, it is possible to generate discriminating sequences for similar genes and to construct different probes for a particular sequence in order to prove hybridization specificity. In studies of monoclonal B-cell lines with immunoglobulin variable region (VH) genes of known nucleic acid sequence, Long et al showed high levels of specificity for 35S labeled oligonucleotide probes.[19] Clones expressing more than 90% sequence homology could be distinguished using ISH.

A variety of protocols are available for radioisotopic ISH methods.[20] When using radioactive probes the highest degree of resolution is obtainable with tritium; however, exposure times are quite long. Although 32P provides short exposure times, resolution is poor. 35S, which provides good resolution with relatively short exposure times, has been the isotope of choice for most recent studies of gene expression. The autoradiographic methods of ISH offer the advantage of quantitative analysis of gene expression based on computerized counting techniques.[21]

Although earlier studies used radioactive probes for ISH analyses, more recent studies, particularly those in clinical laboratories, have used an increasing variety of nonradioactive probes.[22] The advantages of nonradioactive probe-labeling technologies include ease of performance with short procedure and turnaround times, lower reagent costs, longer shelf time, and absence of the need for radioactive containment and disposal facilities. A distinct advantage of nonradioactive methods is their high degree of resolution, which permits localization of DNA at specific chromosomal sites and mRNA in subcellular compartments.[23]

A variety of nonisotopic probes have been used in ISH formats (Table 1). Generally, these probes are less sensitive than corresponding radio-labeled probes and are not useful for the detection of very low abundance mRNAs.[24] At present, both biotin and digoxigenin are used most extensively. Biotin-labeled probes can be prepared by the enzymatic incorporation of biotin-labeled analogues of uridine triphosphate (UTP) into nucleic acids. Sites of hybridized biotin can be visualized by the addition of avidin or streptavidin conjugated to fluorochromes, enzymes (horseradish peroxidase, alkaline phosphatase, glucose oxidase) or colloidal gold particles. Al-

TABLE 1. Nonisotopic Probe Labeling Techniques

Hapten modified probes
Biotin
Photobiotin
Digoxigenin
Bromodeoxyuridine
Acetylaminofluorene
Sulfonation
Mercury trinitrophenyl
Direct labeling probes
Fluorochromes (tetramethylrhodamine, fluorescein thiosemicarbozide)
Enzymes (horseradish peroxidase, alkaline phosphatase)
Antibodies
AntiDNA:RNA or RNA:RNA hybrid antibodies

ternatively, antibiotin antibodies may be used for the detection of hybridized biotin.[24]

Digoxigenin is a steroid hapten that is derived from the cardiac glycoside digoxin.[25] Probes labeled with digoxigenin have higher sensitivities than do corresponding biotinylated probes.[24] Hybridized digoxigenin-labeled probes can be visualized by the use of a sheep antidigoxigenin Fab fragment conjugated to alkaline phosphatase or other reporter molecules.[25,26] Background staining is less problematic than with biotinylated probes because digoxigenin is not present in mammalian cells. This is a particular advantage when studying tissues like liver, which are rich in biotin. The availability of a variety of different probe labels and detection systems allows multiple DNA and RNA targets to be shown simultaneously.[27,28]

In hybridization reactions the hybrid melting temperature (Tm) is defined as the temperature at which 50% of the duplex strands dissociate or melt. Factors that influence duplex stability include the ionic strength of the reaction, the proportion of guanine-cytosine (G:C) base pairs (BPs), probe length, proportion of mismatched bases between the target and the probe, and the concentration of formamide in the hybridizing solution.[12,15] Formamide is added in these procedures to decrease the melting temperature of the hybrids. The term stringency refers to the degree to which reaction conditions favor duplex dissociation. High temperatures, high formamide concentrations, and low ionic strength provide highly stringent conditions. Under highly stringent conditions duplexes that have a high degree of homology will have a greater degree of stability than will duplexes with a low degree of homology. In general, high degrees of specificity can be obtained by increasing the stringency.

The results of ISH are subject to the influence of numerous variables that must be controlled rigorously if reliable and reproducible results are to be obtained.[14,15] Generally, detection of gene expression by ISH should include the use of alternative molecular methods including Northern blot analysis and PCR. Of particular value as a positive control is the use of normal or neoplastic tissue known to highly express a specific gene or the use of cell lines genetically engineered to express the gene of interest. Cells that do not express the gene of interest present important negative controls. Cells that express a related but distinct gene also provide important controls of specificity. Other controls include the use of probes that have been prehybridized with their complementary sequences, hybridization with nonspecific vector sequences, and in the case of antisense RNA probes the use of the corresponding sense probe that should fail to hybridize with the mRNA of interest.[14,15]

GENE EXPRESSION

In situ hybridization has emerged as a remarkably powerful tool for the assessment of gene expression in tumor cells at the cellular level. In contrast to immunohistochemistry, which is dependent on the protein content of cells, hybridization analyses offer the advantage of identifying cells on the basis of their contents of specific mRNAs encoding the products of interest. In situ hybridization expands the results of conventional molecular techniques, such as Northern blot analysis, in much the same way that immunohistochemistry expands Western blot assays. Both immunohistochemistry and ISH provide data on individual cells rather than on an average of the total cellular population. Additionally, changes in mRNA levels as determined by Northern blot and quantitative ISH assays can more closely correlate with functional activity than the results of immunohistochemistry for the demonstration of translational products.[29,30] High levels of a particular product, for example, as determined by immunohistochemistry could represent increased synthesis or decreased synthesis with storage of the particular product within the cells. In situ hybridization therefore provides a particularly critical approach for the analysis of heterogeneity in tumors that typically contain cells at different phases of neoplastic progression, at multiple levels of differentiation, and at different levels of functional activity.

The techniques of ISH have proven to be effective for the analysis of oncogene overexpression in a variety of different tumor types. Cohen et al, for example, established the feasibility of using this approach for analysis of N-*myc* oncogene overexpression in a series of neuroblastomas studied previously by Southern analysis.[31] There was an excellent correlation between the two approaches, but ISH offered the advantage of detecting heterogeneity with respect to N-*myc* mRNA expression within different areas of the tumor. Although these investigators did not detect hybridization signals in mature ganglionic cells, Fabbretti et al have shown N-*myc* overexpression both in neuroblastic and ganglionic cells in two cases of stroma-rich ganglioneuroblastoma.[32] Leong et al have published a protocol that permits the simultaneous analysis of N-*myc* overexpression and deletion of 1p using different fluorophores both in neuroblastoma cell lines and in intact tumors.[28]

These methods also have been used for the localization of mRNAs encoding a number of other oncogenes, including c-*myc*, bcl-2, and Her 2/*neu*.[33-36] In a series of breast-carcinoma cases reported by Naber et al, there was a close correlation between the level of Her 2/*neu* mRNA expression as determined by ISH and Northern blot analysis and the extent of Her 2/*neu* protein staining by immunohistochemistry.[35] Studies reported by Murty et al using radiolabeled antisense oligonucleotide probes in Northern blot and ISH analyses have shown that bcl-2 expression was increased two to three times in follicular lymphomas with t(14;18) and that *myc* expression was increased two to four times in high-grade lymphomas with t(14;18) as compared with reactive lymphoid cells or lymphoma cells without translocations.[34]

In situ hybridization analyses also have been used extensively in studies of endocrine neoplasms.[30,37] These studies have shown the presence of hormone messenger RNAs independent of storage or secretory ability. For example, endocrine cells that are secreting their products constitutively may give only equivocally positive or negative immunohistochemical signals with strongly positive hybridization signals.[30] Extensive posttranslational processing also may result in situations in which immunohistochemical results are negative but ISH results are positive. In such instances antibodies raised against natural or synthetic hormones may fail to react with epitopes on hormone precursors that may have been modified extensively within the endoplasmic reticulum, Golgi regions, or secretory granules themselves. This phenomenon may occur in certain actively-

synthesizing endocrine neoplasms such as insulinomas, which secrete considerably more proinsulin than insulin. Extensive intracellular degradation of peptides may also lead to instances in which there are negative immunohistochemical results but positive hybridization signals. Finally, ISH is the method of choice to differentiate de novo synthesis from hormonal uptake of peptides.

The practical advantages of ISH as compared with immunohistochemical analysis of endocrine tumors have been shown in several studies. For example, small cell bronchogenic carcinomas, which commonly are negative for chromogranin proteins, frequently give a positive signal when chromogranin mRNA probes are used in ISH formats.[38,39] In addition, both pro-opiomelanocortin[40] and gastrin-releasing peptide[41] mRNAs can be shown more consistently than the corresponding peptides in these tumors. The studies of Lloyd et al have shown that pituitary null-cell adenomas and gonadotroph adenomas are closely related neoplasms on the basis of in situ demonstration of mRNAs for the alpha subunit of glycoprotein hormones in the majority of null-cell tumors.[42]

Studies of calcitonin and calcitonin gene-related peptide (CGRP) expression in medullary thyroid carcinoma and C-cell hyperplasia have demonstrated that the carcinoma cells show an increase in the content of CGRP mRNA and peptide relative to that of calcitonin, suggesting a defect in the control of transcript processing.[43] Moreover, there is a decrease in the ratio of calcitonin peptide to its corresponding mRNA as compared with that observed in normal C cells. These observations suggest a tumor-associated defect in calcitonin synthesis or storage.

The methods of ISH also provide powerful tools for the demonstration of hormone receptors in target cells.[44] Comparative studies of immunohistochemical and ISH techniques for the demonstration of estrogen receptor (ER) have shown a higher sensitivity for ISH methods even in formalin-fixed, paraffin-embedded samples of breast carcinoma.[45,46] The clinical significance of breast carcinomas that are ER mRNA positive but ER protein negative requires further study. Androgen receptor mRNA also has been demonstrated successfully in situ hybridization methods.[47]

A wide variety of messenger RNAs encoding products of interest and importance in understanding neoplasms have been successfully localized with ISH methods. These include tumor-associated markers, such as carcinoembryonic antigen (CEA),[48] growth factors and growth-factor receptors,[49-51] cytokines,[52] T-cell receptor β-chain,[53] and immunoglobulin (Ig) light chains.[54-56] Although the diagnostic use of these approaches has not been fully exploited, some of them hold considerable potential. Several groups of investigators, for example, have localized immunoglobulin light chain mRNAs both in hyperplastic and neoplastic lymphoproliferative processes. Although the sensitivity of these methods is less than that obtained by studies of the corresponding proteins, the problem of intercellular immunoglobulin staining is virtually eliminated. Moreover, the distinction of cells that have the capacity to synthesize immunoglobulins from those in which immunoglobulins are absorbed can be made readily.

There are numerous additional applications of ISH analyses of gene expression in tumor pathology. For example, Chou et al have described an ISH method for determining proliferative activity of cells based on the demonstration of cellular mRNA levels of histone H3, an S-phase specific gene product.[57] This technique has been applied successfully to the analysis of formalin-fixed, paraffin-embedded samples. In situ hybridization methods also have proven to be of value in the characterization of tumor invasion. For example, the 72kD and 92kD type IV collagenase mRNAs have been localized to stromal fibroblasts and macrophages, respectively, adjacent to the invading fronts of colonic adenocarcinomas. Gray et al have made similar observations and have noted that eosinophils are the predominant producer cell of interstitial collagenase.[59] Eosinophils also express the type IV collagenase mRNA in basal cell carcinomas suggesting that eosinophils may be capable of producing multiple metalloproteinases.[60] These observations highlight the fact that several nonepithelial cell types participate in the production of factors involved in extracellular proteolysis during cancer invasion.

FUTURE PROSPECTS

Although ISH techniques are highly sensitive approaches for the study of genes and gene expression, the sensitivity of these methods can be enhanced substantially by combination with PCR.[61,62] Labeled nucleotides can be incorporated directly into in situ amplificants (direct in situ PCR) or, alternatively, ISH can be performed after in situ amplification using labeled oligonucleotide probes (indirect in situ PCR). These approaches have been used successfully for the detection of low copy number viral DNAs, single-copy genes, and immunoglobulin gene rearrangements in cell suspensions and in cytospins.[63] Nuovo et al have introduced an important modification of in situ PCR in which the cells are heated before the addition of Taq polymerase or primers until the reaction temperature approaches 80°C (Hot-Start method).[63,64] This technique, which has been used in formalin-fixed, paraffin-embedded samples, can detect as few as one human papilloma virus (HPV)-16 copy per cell.[62] A similar approach has been used successfully for the identification of human immunodeficiency virus (HIV) 1 DNA.[66]

The use of in situ PCR also has been extended to studies of RNA after the in situ synthesis of the corresponding cDNA through the use of reverse transcriptase. This approach has been used successfully for the detection of RNA viruses[67,68] and for the identification of granzyme A and perforin mRNAs in cytotoxic T lymphocytes.[69] Although there have been few applications of this method in diagnostic laboratories, the ability to amplify mRNA in situ promises to be an extraordinarily sensitive approach for the analysis of gene expression.

APPENDIX

Abbreviations: ISH, in situ hybridization; mRNA, messenger RNA; PCR, polymerase chain reaction; cDNA, complimentary DNA; cRNA, complimentary RNA; APEX, amplified primer extension; VH, variable region; UTP, uridine triphosphate; Tm, hybrid melting temperature; BP, base pair; CGRP, calcitonin gene-related peptide; ER, estrogen receptor; Ig, immunoglobulin; CEA, carcinoembryonic antigen; HPV, human papilloma virus; FISH, fluorescence in situ hybridization; RT, reverse transcription.

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